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DOI:

[10.1021/acs.molpharmaceut.8b00381](https://doi.org/10.1021/acs.molpharmaceut.8b00381)

Document Version

Peer reviewed version

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Citation for published version (APA):

Jorgensen, C., & Domene, C. (2018). Location and character of volatile general anesthetics binding sites in the transmembrane domain of TRPV1. *Molecular Pharmaceutics*. Advance online publication.

<https://doi.org/10.1021/acs.molpharmaceut.8b00381>

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Location and character of volatile general anesthetics binding sites in the transmembrane domain of TRPV1

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Abstract

It has been proposed that general anesthesia results from direct multisite interactions with multiple and diverse ion channels in the brain. An understanding of the mechanisms by which general anesthetics modulate ion channels is essential to clarify their underlying behavior and their role in reversible immobilization and amnesia. Despite the fact that volatile general anesthetics are drugs that primarily induce insensitivity to pain, they have been reported to sensitize and activate the vanilloid-1 receptor, TRPV1, which is known to mediate the response of the nervous system to certain harmful stimuli, and which plays a crucial role in the pain pathway. Currently, the mechanism of action of anesthetics is unknown and the precise molecular sites of interaction have not been identified. Here, using $\sim 2.5 \mu\text{s}$ of classical molecular dynamics simulations and metadynamics, we explore these enigmas. Binding sites are identified and the strength of the association is further characterized using alchemical free-energy calculations. Anesthetic binding/unbinding proceeds primarily through a membrane-embedded pathway, and subsequently, a complex scenario is established involving multiple binding sites featuring single or multiple occupancy states of two small volatile drugs. One of the five anesthetic binding sites reported was previously identified experimentally, and another one importantly, is identical to that of capsaicin, one of the chemical stimuli that activate TRPV1. However, in contrast to capsaicin, isoflurane and chloroform binding free-energies render modest to no association compared to capsaicin, suggesting a different activation mechanism. Uncovering chloroform and isoflurane modulatory sites will further our understanding of the TRPV1 molecular machinery and open the possibility of developing site-specific drugs.

Keywords:

anesthesia; molecular dynamics simulations; docking; funnel metadynamics; TRP channels

Introduction

It has been proposed that general anesthesia results from direct multisite interactions with multiple and diverse ion channels in the brain.¹ Experimental findings of volatile general anesthetics binding to ion channels comes from structural work, where crystal structures of membrane proteins were obtained in complex with volatile general anesthetics,²⁻⁵ as well as mutagenesis⁶ and photolabelling⁷ studies. In particular, evidence of a modulation mechanism for anesthetics on both the nAChR (nicotinic acetylcholine) and GABAA receptors involving multiple binding sites has been described experimentally.⁸⁻¹⁰ In parallel, computational studies have yielded insights into volatile general anesthetics binding in membrane proteins,¹¹⁻¹⁴ including simulations using a supersaturated isoflurane concentration suggesting that the modulation of nAChR and GLIC ion channels, occurs by binding to multiple sites.¹¹ In sodium channels, microsecond simulations of NavAb revealed a similar multiple-binding site model for local anesthetics benzocaine and the anti-seizure drug phenytoin, and yielded two drug-access pathways into the pore, a lipophilic access pathway through lateral fenestrations, and an aqueous pathway through the intracellular activation gate for local anesthetic benzocaine¹² as well as volatile general anesthetics isoflurane and sevoflurane, which have been shown to modulate the gating of the prokaryotic Na⁺ channel NaChBac by increasing forward activation rates.¹⁵⁻¹⁶ At present, there is growing evidence that most general and local anesthetics as well as some analgesics also activate or sensitize nociceptors via TRPA1 or TRPV1 ion channels.¹⁷⁻¹⁸ Recently, a putative binding site for the general anesthetic propofol has been identified in the TRPA1 ion channel using mutagenesis studies,¹⁹ near the cavity lined by residues S876, M915, and M956 from the S5 and S6 transmembrane helices. Further work by photolabeling has yielded supporting evidence for this site, highlighting the residues the V954 and E969 from the S6 transmembrane helix as crucial in the activation of TRPA1 by propofol.²⁰ Activation and sensitization of TRPV1 induced by local anesthetics is thought to involve a domain that is similar but not identical to the vanilloid-binding domain, the area of the protein that interacts with its agonist.¹⁷

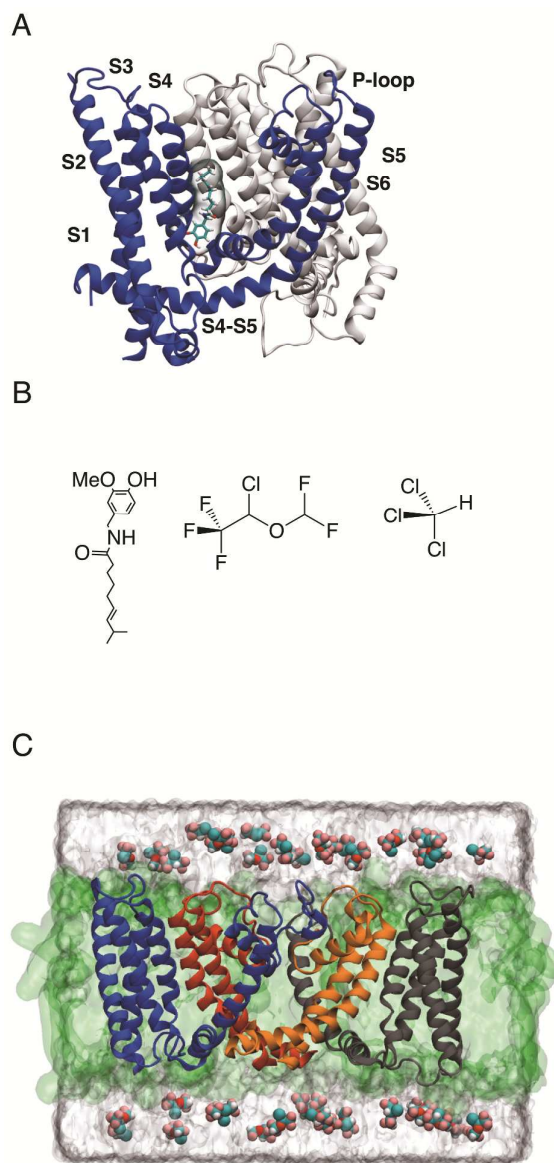
Volatile general anesthetics (VGAs) span a group of chemicals that are able to reversibly inhibit the central nervous system activity, rendering patients unresponsive to stimuli in contrast to local anesthetics. Little is known about the molecular targets of inhaled anesthetics to relate their effect to pharmacology. However, their binding sites are known to be hydrophobic with some polar character and with sufficiently general features to be widespread.²¹ Volatile general anesthetics including isoflurane (1,1,1-trifluoro-2-chloro-2-(difluoromethoxy)-ethane), chloroform, propofol, and halothane are reported to bind directly to ion channels.^{3-4, 22-23} In contrast to their inhibitory effects on the central nervous system, several volatile general anesthetics are known to activate or sensitize the signaling of peripheral nociceptive (pain-sensing) neurons²⁴ which gives origin to their pungency or the condition of having a strong and sharp smell or taste that can be unpleasant.

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3 The transient receptor potential (TRP) ion channels constitute a large and diverse family of non-
4 selective cation channels, found in yeast and widespread in the animal kingdom.²⁵ These channels are
5 expressed in excitable and non-excitable cell tissue, playing a critical role in sensory physiology by
6 acting at the cell level, including synaptic activity or hormone secretion, and at organism levels (tactile,
7 hearing, taste, olfaction, vision and thermal sensation).²⁵⁻²⁶ TRP channels display multifunctional and
8 polymodal behavior in their regulation and interactions. In particular, the vanilloid-1 channel (TRPV1),
9 arguably the best-characterized member of the vertebrate TRP family, is activated by a diverse array of
10 physical and chemical stimuli such as noxious heat, inflammatory agents, such as extracellular protons
11 and lipids, or capsaicin, the active compound of chili peppers that elicits burning pain.²⁷ Upon capsaicin
12 stimulation, the channel undergoes pore dilation, in which its selectivity for large cations over sodium
13 ions is increased. While a single capsaicin-bound subunit was sufficient to achieve maximal open-
14 channel lifetime, all four proton-binding sites were required. Therefore, the pain receptor TRPV1 was
15 reported to display agonist-dependent activation stoichiometry.²⁸

22 Chloroform became the most popular volatile general anesthetic of the 19th century, before being
23 abandoned because of its low therapeutic index,²³ but has recently regained interest as having been
24 reported to activate the TRPV1 ion channel at millimolar concentrations using a similar mechanism as
25 isoflurane. In particular, experimental data has suggested that TRPV1 is directly activated by
26 chloroform, and none of the other heat-activated channels (TRPV2, TRPV3 or TRPV4) are activated by
27 chloroform at clinical concentrations that are reported to elicit a robust activation of TRPV1.²³

31 Chloroform at a concentration of ~6.3 mM had solely an additive effect on channel activation when
32 administered in the presence of capsaicin concentrations of ≤ 5 nM, while at concentrations ≥ 10 nM
33 capsaicin yielded indistinguishable activation in the presence or absence of chloroform.²³ It has been
34 postulated that residue E600 is required for proton and volatile general anesthetics activation, and Y653
35 is required for heat and volatile general anesthetics activation of TRPV1,²³ and that these two residues,
36 E600 and Y653, might facilitate channel activation.²⁹ Recent structural and biophysical studies have
37 resolved the atomic structures of some of the members of the TRP family primarily by cryo-electron
38 microscopy.^{27,29} These structural models provide an excellent starting point for the mechanistic study of
39 anesthetic interactions and binding at the molecular level.

45 From the cryo-EM structure, the TRPV1 channel architecture consists of a tetrameric assembly of four
46 monomeric subunits, analogous to those of voltage-gated sodium and potassium channels. Each subunit
47 consists of six trans-membrane α -helices (S1–S6) and an S5-P-S6 pore loop–helix (Figure 1.A).
48 Previously, computational studies reported cation binding sites in the pore domain of TRPV1,^{30,31} in
49 particular confirming the capsaicin binding poses (Figure 1.A).³² Here, we attempt to explore the nature
50 of the binding sites evoked by two volatile general anesthetics in TRPV1, chloroform and isoflurane
51 (Figure 1.B), compare them with capsaicin, and narrow down putative interaction sites hinted in
52 experimental studies. The system setup is represented in Figure 1.C.



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Figure 1. (A) Two TRPV1 subunits shown in blue and white, respectively indicating the site where capsaicin binds reported in structural studies.³² Capsaicin is shown in licorice representation, and the structural elements of one of the monomers are labelled (helices S1 to S6 and P-loop connecting helices S5 and S6). (B) Chemical structure of capsaicin, isoflurane and chloroform respectively. (C) Simulation setup for the TRPV1 transmembrane region embedded in a POPC membrane shown in green, and with an initial random distribution of volatile anesthetics in solution in the bulk.

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Materials and Methods

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System set-up

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The atomic model of the apo open-activated state of the transmembrane region of the vanilloid receptor 1 (TRPV1) was retrieved from the protein data bank, PDB ID 3J5Q,²⁹ at resolution 3.4 Å (residues V430 to V686). The set-up was done with CHARMM-GUI (<http://www.charmm-gui.org>).³³ The N-terminus was acetylated and the C-terminus was methylated. The CHARMM36 force field with CMAP corrections was used for the protein³⁴⁻³⁵ and lipids³⁶ together with the TIP3P model for water molecules.³⁷ CHARMM force field parameters for isoflurane³⁸ and chloroform³⁹ were used. Default

ionization states were used for the protein on the basis of PropKa calculations.⁴⁰ The protein was embedded in a pre-equilibrated lipid bilayer of 240 POPC (1-palmitoyl, 2-oleoyl-sn-glycero-3-phosphocholine) lipids using the replacement method of CHARMM-GUI, with 120 lipids in the upper-layer, and 120 lipids in the lower layer, with the axis aligned to the bilayer normal. The system was embedded in a rectangular water box of dimensions (110, 110, 85) Å³. A 150 mM KCl solution was added to neutralize the system. The full TRPV1 pore system (residues V430-V686) comprised ~90,000 atoms, while the pore model (E570-V686) comprised ~50,000 atoms. The total concentration of isoflurane and chloroform added independently was 8 mM to facilitate sufficient statistics.

Equilibration was done using NAMD version 2.9⁴¹ starting with 5,000 steps of steepest-descent minimization, 75 ps of dynamics in the NVT ensemble with restraints on the backbone with a time-step of 1 fs, followed by 300 ps of dynamics in the NVT ensemble with restraints on the backbone with a time-step of 2 fs. Finally, the system was evolved 1.0 ns in the NPT ensemble. The output of the MD equilibration was used as the starting point for two subsequent sets of MD production runs with (i) 40 isoflurane or (ii) 40 chloroform molecules (Figure 1.C). Production runs of the flooding simulations were performed in the NPT ensemble. A summary of the simulations performed is reported in the Supplementary Material Table S1. The particle mesh Ewald (PME) algorithm was used for the evaluation of electrostatics interactions beyond 12 Å, with a PME grid spacing of 1 Å, and NAMD defaults for spline and κ values.⁴² A cut-off at 12 Å was applied to non-bonded forces. Both electrostatics and Van der Waals forces were smoothly switched off between the cut-off distance of 12 Å and the switching distance of 10 Å, using the default NAMD switching function. A Verlet neighbor list with pairlist distance of 16 Å was used to only evaluate non-bonded neighboring forces within the pairlist distance⁴³. The lengths of covalent bonds involving hydrogen atoms were constrained by the SETTLE algorithm⁴⁴ in order to be able to use a 2-fs time-step. The multi time step algorithm Verlet-I/r-RESPA^{43,45} was used to integrate the equations of motion. Non-bonded short-range forces were computed for each time step, while long-range electrostatic forces were updated every 2 time steps. The pressure was kept at 1.013 atm by the Nosé-Hoover Langevin piston⁴⁶⁻⁴⁸ with a damping time constant of 25 fs and a period of 50 fs. The temperature was maintained at 303.15 K by coupling the system to a Langevin thermostat, with a damping coefficient of 1 ps⁻¹.

Anesthetic binding sites from volumetric data maps

Anesthetic binding sites in the two 500-ns simulations were characterized from volumetric data maps of isoflurane and chloroform occupancy calculated with the Volmap tool of VMD.⁴⁹ This tool calculates volumetric maps, which are 3D grids that have a value assigned at each grid point, based on the atomic coordinates of a specified atom selection using either a density or occupancy criterion. In occupancy maps, each grid point is assigned to be 1 if it contains one or more atoms of the selection or 0 if it does not. When averaged over all the frames of the trajectory, this map contains the fractional occupancy of that grid point. Here, occupancy maps were calculated based on the selection of all 40 isoflurane or 40

chloroform molecules respectively from each frame, and were averaged over the entire trajectory. The fractional occupancy out of 100% was represented as iso-20% contour surface maps in Figures 3 and 4.

Free energy of insertion of anesthetics in the membrane

In order to estimate the partition free energies of isoflurane and chloroform in the membrane, estimates of the free energy of insertion in the POPC lipid bilayer were calculated using metadynamics⁵⁰ with a funnel-restrained potential,⁵¹ enabling the exploration of both the unbound bulk and lipid-partitioned states. An equilibrated bilayer of 100 POPC molecules with just one anesthetic molecule was set up, with a 0.15 mM background KCl. A funnel-restrained well-tempered metadynamics⁵⁰ calculation using the collective variable ξ_1 as the distance along the Z-axis was used, where $\xi_1 = 0 \text{ \AA}$ constitutes the membrane centre and $\xi_1 = 35 \text{ \AA}$ is the bulk solution. In addition, ξ_2 is the radial distance of the drug in the funnel, as measured from the Z-axis which corresponds to the transversal funnel degree of freedom. This simulation was performed using Plumed 1.3⁵² with NAMD 2.9, with a Gaussian of width 0.2 \AA and initial height 0.12 kcal/mol deposited every 1 ps using a bias factor of 12. The funnel-restrained potential is a combination of a cone restraint which includes the external side of the membrane, and a cylindrical part which is directed toward the solvent.⁵¹ The alpha angle, α , denoting the funnel angle from the normal axis was set to 0.55 rad (31.5°) and Z_{cc} the funnel height was set to 35 \AA , placed at the center of the membrane. A geometric correction term (Equation 1) is added to the free-energy, ΔG , to give the free-energy of translocation from the bulk to the membrane, ΔG° , by computing the geometric contribution from the bulk cylinder of radius $R_{cyl} = 1 \text{ \AA}$, scaled by the standard concentration $C^0 = 1 \text{ mol dm}^{-3} = 1/1661 \text{ particles \AA}^{-3}$.

$$\Delta G^\circ = \Delta G - \frac{1}{\beta} \ln(\pi R_{cyl}^2 C^0) \quad \text{Equation 1}$$

An error analysis was performed to obtain the fluctuation in the ΔG for the translocation between aqueous and membrane phase by calculating the time evolution of the ΔG between the aqueous ($25 \text{ \AA} < \xi < 30 \text{ \AA}$) and the membrane center ($7 \text{ \AA} < \xi < 12 \text{ \AA}$) potential of mean force. This value was averaged for the plateau which corresponds to the last 100 ns of the simulation, and a standard deviation is reported such that the translocation is $\Delta G \pm \sigma$.

Standard free energy of binding of anesthetics to TRPV1

The free energy of binding, ΔG_{bind} , of each anesthetic to TRPV1 was calculated and compared to the binding energy of the TRPV1 agonist capsaicin,³² starting from the pose observed when bound in site II'. The value of the free energy of binding of capsaicin to TRPV1 was reported³² to be $-10.6 \pm 1.7 \text{ kcal mol}^{-1}$ obtained using the method by Gumbart et al.⁵³ A schematic representation of the cycle is presented in Figure S1.A and shown exemplified by isoflurane in Figure S1.B. This method gives the change in the free energy of binding, ΔG_{bind} , but does not reveal the cost of translocating the anesthetic to the binding site, and the method relies upon alchemical free-energy perturbation transformations

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4 combined with geometrical restraints on the anesthetics to avoid the wandering ligand problem at the
5 end of the decoupling or beginning of coupling simulations.⁵⁴⁻⁵⁵ Reversible coupling of the anesthetic to
6 its environment, either coupling to the TRPV1 pocket (bound) state or decoupling in the bulk aqueous
7 solution (unbound state) was performed bi-directionally using the free energy perturbation (FEP)
8 method. The order parameter λ was evenly divided into 16 windows of width equal to 0.05 in the range
9 0.1 to 0.9, plus 40 windows of width equal to 0.005, in the range 0 to 0.1 and 0.9 to 1.0. Each window
10 consisted of 200,000 data collection steps (0.4 ns) preceded by 50,000 equilibration steps (0.1 ns). The
11 total simulation time was 28 ns per coupling/decoupling cycle, totaling 54 ns for the bound state and 54
12 ns for the unbound state. The total simulation time employed for the alchemical FEP calculations was
13 216 ns.
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18 The convergence of the alchemical calculations was evaluated by analyzing the overlap of the ΔU
19 distributions for the forward (ΔU_0) and backward (ΔU_1) calculations, per window (Figure S2). The
20 statistical data was combined by means of the Bennett acceptance ratio (BAR)⁵⁶ which provides a
21 maximum-likelihood estimator of the free-energy change.⁵⁷ The ParseFEP tool⁵⁸ implemented in
22 VMD⁴⁹ was used to analyze the results. To prevent the wandering ligand problem, the conformation of
23 anesthetic was restrained during the free-energy calculations and each calculation was based on the
24 central cluster node from the MD binding site II'' (capsaicin binding site) occupied in one of the four
25 TRPV1 subunits. Geometrical restraints were defined based on three invariant protein reference sites
26 (P1, P2, P3). These sites were defined as the center of mass (COM) of heavy atoms of residues A566,
27 F582 and L664. In addition, three reference sites in the anesthetic molecules (L1, L2, L3) were defined.
28 These sites correspond to the center of mass of selected pairs of atoms in isoflurane or chloroform. In
29 total, six sites were used to define the harmonic restraints u_r (distance P1-L1), u_θ (angle P2-P1-L1),
30 and u_ϕ (dihedral P3-P2-P1-L1) used to fix the position of the anesthetic molecule in the protein
31 pocket. U_θ (angle P1-L1-L2), u_ϕ (dihedral P1-L1-L2-L3), and u_ψ (dihedral P2-P1-L1-L2) were
32 used to fix the anesthetic orientation. Finally, a harmonic RMSD restraint on the conformation of the
33 anesthetic molecule (u_c) ensured the pose was fully fixed. To estimate the contribution to the free energy
34 of binding as a result of the restraints, thermodynamic integration (TI) simulations were performed
35 coupling the force constant of each harmonic potential to the order parameter λ in a 12-point grid. The
36 gradient of the potential energy with respect to the collective variable was calculated from MD
37 simulations at each value of λ . Each simulation consisted of 200,000 data collection steps (0.4 ns), after
38 50,000 steps (0.1 ns) of equilibration. Scaling of the force constants was also performed bi-
39 directionally, and the free energy contribution of the restraints was retrieved averaging both
40 contributions. The error was estimated as the maximum hysteresis between forward and backward
41 calculations.
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54 **Docking procedure**

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2 Docking of anesthetics was performed using Autodock (v4.2) on the full TRPV1 pore (V430-V686).⁵⁹
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4 Two dockings were performed, one based on the holo-TRPV1 trajectory (PDB id 3J5Q; Resolution 3.8)
5 and the second based on an apo-TRPV1 trajectory (PDB id 3J5P; Resolution 3.4 Å). For trajectories of
6 either the apo or holo system, the first 50 ns of equilibration were discarded after inspections of the
7 protein backbone RMSD plot. Subsequently, 500 snapshots at intervals of 0.2 ns were extracted for
8 docking from the holo system, and 300 snapshots at intervals of 0.2 ns from the apo system. A global
9 search was carried out with a large box centered on the protein and employing 140 x 140 x 110 grid
10 points with default grid spacing of 0.375 Å. For each docking snapshot, 10 solutions were requested.
11 Altogether, 5,000 and 3,000 docking poses were generated respectively, which were later clustered
12 according to the RMSD of the ligand using a RMSD < 2.5 Å criteria per cluster.
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18 **Results**

19 **Partition of anesthetics in the lipid bilayer**

20 An interaction between inhalational anesthetics and proteins was first suggested by Claude Bernard in
21 1875,⁶⁰ and subsequently by Moore and Roaf in 1904 and 1905⁶¹⁻⁶² (see reference [21] for a detailed
22 historical account). In contrast, Meyer, Overton, and others, proposed that the lipid membrane of the
23 cell was generally to be the primary site of action of anesthetics.⁶³⁻⁶⁴ Recent work continues to suggest a
24 membrane-mediated mechanism whereby chemical compounds may modify the properties of the cell
25 membrane, which in turn, would alter the properties of the proteins embedded in the cell membrane.
26 Alternatively, anesthetics would need to cross the water-membrane interphase until they reach specific
27 sites in the protein and as a result of these interactions the protein gating and permeation properties will
28 be altered. Currently, the favoured hypothesis proposes that general anesthesia results from direct
29 multisite interactions with multiple and diverse ion channels in the brain as gradually a number of
30 important molecular targets have emerged. In this respect, how volatile general anesthetics act at the
31 molecular level is becoming clearer.
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39 Two 0.5- μ s MD flooding simulations of either isoflurane or chloroform partitioning into a fully
40 solvated TRPV1 transmembrane domain inserted in a POPC bilayer were performed. Partitioning
41 events observed are reported in Figure 2 and a description of the binding sites to TRPV1 in Figure
42 3. The free energy of translocation is reported in Figure 4, and the free-energy of membrane partitioning
43 from metadynamics is reported in Figure 5. Overall, the protein is stable during the simulation time
44 even in the presence of a high concentration of anesthetics as illustrated from the analysis of the protein
45 all-atom backbone root-mean-square deviation that renders values of 2.2 ± 0.2 Å and 2.7 ± 0.3 Å with
46 fluorine and cholesterol respectively (Supplementary Material Figure S3 and Table S1). These values
47 are within the resolution of the model structure employed indicating that equilibration was achieved.
48 Chloroform and isoflurane can affect the TRPV1 ion channel by partitioning into the plasma membrane,
49 a process facilitated by hydrophobic interactions with the inner-membrane core, and then can modify
50 the properties of the bilayer, or alternatively, they can reach the transmembrane region of the protein
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and compete with capsaicin and other activators. In this context, while both chloroform and isoflurane have moderate lipophilicity⁶⁵ (logP 2.0 and 2.1, respectively), chloroform possesses a dipole moment (9.5 Å³) and has been found to display a slight preference for the membrane core.⁶⁶ Isoflurane, in contrast, is a non-hydrophobic anesthetic^{38,67} which readily partitions into the lipophilic phase, but shows a preference for the interfacial regions, with some studies suggesting that it does not readily accumulate in the hydrophobic membrane core.^{11,68} The behavior of other TRPV1 agonists such as camphor (logP 2.2) is similar to isoflurane showing preference for the interface which means its therapeutic dose is higher than that for other agonists.⁶⁹ Strikingly, despite the relatively high concentration required for activation and potentiation of TRPV1, the effects of camphor were found to be rapidly and readily reversible and a mechanical rather than a biochemical link to the pore gating machinery was proposed.⁶⁹

In the current study, both anesthetics partition readily into the membrane phase (Figure 2.A) reaching 95% and 90% for isoflurane and chloroform respectively after 200 ns of simulation time (Figure 2.B). No distortion of the lipid membrane was observed upon partitioning as had already been reported in earlier simulations of isoflurane and similar VGAs.⁷⁰ On the whole, the lipophilic entry route is the primary access mode for these anesthetics to the protein binding sites as illustrated for two diffusion pathways of membrane-partitioned isoflurane and chloroform monitored by the ligand RMSD over trajectory time (Figure 2.C) and depicted as iso-contour trajectory probability in Figure 2.D. Only 5% of isoflurane molecules and 10% of chloroform do not reach their binding sites via membrane partitioning. The secondary pathway (Figure 2.A) is a direct entry into the protein from the bulk (Figure 2.A).

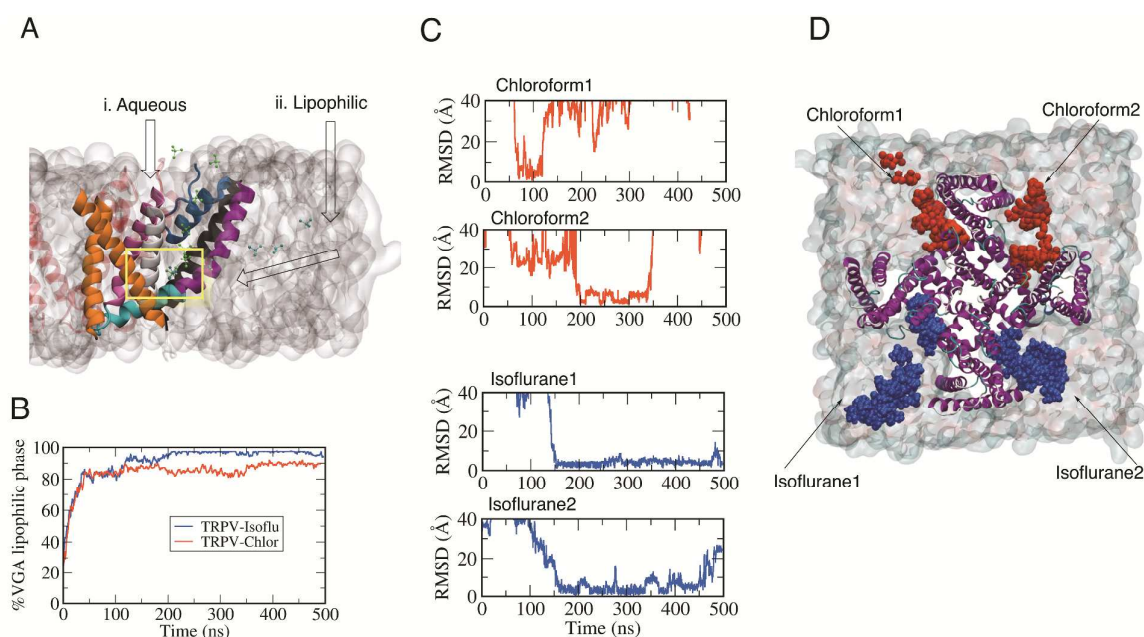


Figure 2. (A) Schematic comparison of the lipophilic and aqueous entry routes into the site II'. The lipid membrane is depicted in grey color and one out of the four monomers composing the transmembrane domain of the ion channels is depicted

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2 in ribbon representation with each transmembrane helix colored differently. (B) Percentage (%) of anesthetics partitioning into
3 the membrane for isoflurane (color code blue; plateau 95%) and chloroform (color code red; plateau 90%) as a function of
4 simulation time. (C) Evolution of the ligand RMSD with respect to its bound pose in the capsaicin site (II') for two isoflurane
5 (blue) and two chloroform (red) molecules. Each molecule is labeled 1 or 2. (D) Illustration of the entry pathway through the
6 membrane observed for each of the two chloroform and isoflurane molecules considered, looking from above the lipid
7 membrane and indicated by arrows. Please note that this illustration was produced from a superposition of frames from two
8 independent simulations.

9 Upon reaching the inner transmembrane region, five binding sites were identified using iso-contour
10 projections (Figure 3.A and B): (a) a pore site, site I (Figure 3.C), (b) the same site capsaicin occupies,
11 site II' (Figure 3.D), (c) two side-pocket sites II and III, and finally (e) site IV, within the outer helices
12 (Figure 3.E). The protein-anesthetic contacts identified are described in detailed in Table S2 and are
13 graphically summarized in for three key sites (Figure 3.C,D,E). The binding sites were validated by
14 projections of the chloroform free-energy surface (Figure 4.A) compared to the iso-contour probabilities
15 (Figure 4.B) and the same for isoflurane (Figure 4.C to 4.D). The stability of the binding poses is
16 characterized by the ligand root mean square deviation relative to the binding pose in each site, as
17 illustrated for representative anesthetic trajectories in Figure 2.C and in full in Supplementary Material
18 Figure S4. The difference in interactions between the protein and both anesthetics suggests chloroform
19 and isoflurane bind differently to TRPV1. The difference can be ascribed to the smaller size of
20 chloroform which allows for sampling regions that are not accessible to isoflurane, resulting in two
21 additional sites accessible to chloroform, site II and III, which are variants of the side-pocket site II' of
22 capsaicin. Site II is flanked by helices S5, S6 and S6' from a different subunit which resembles the
23 propofol binding site recently reported for TRPA1.²⁰
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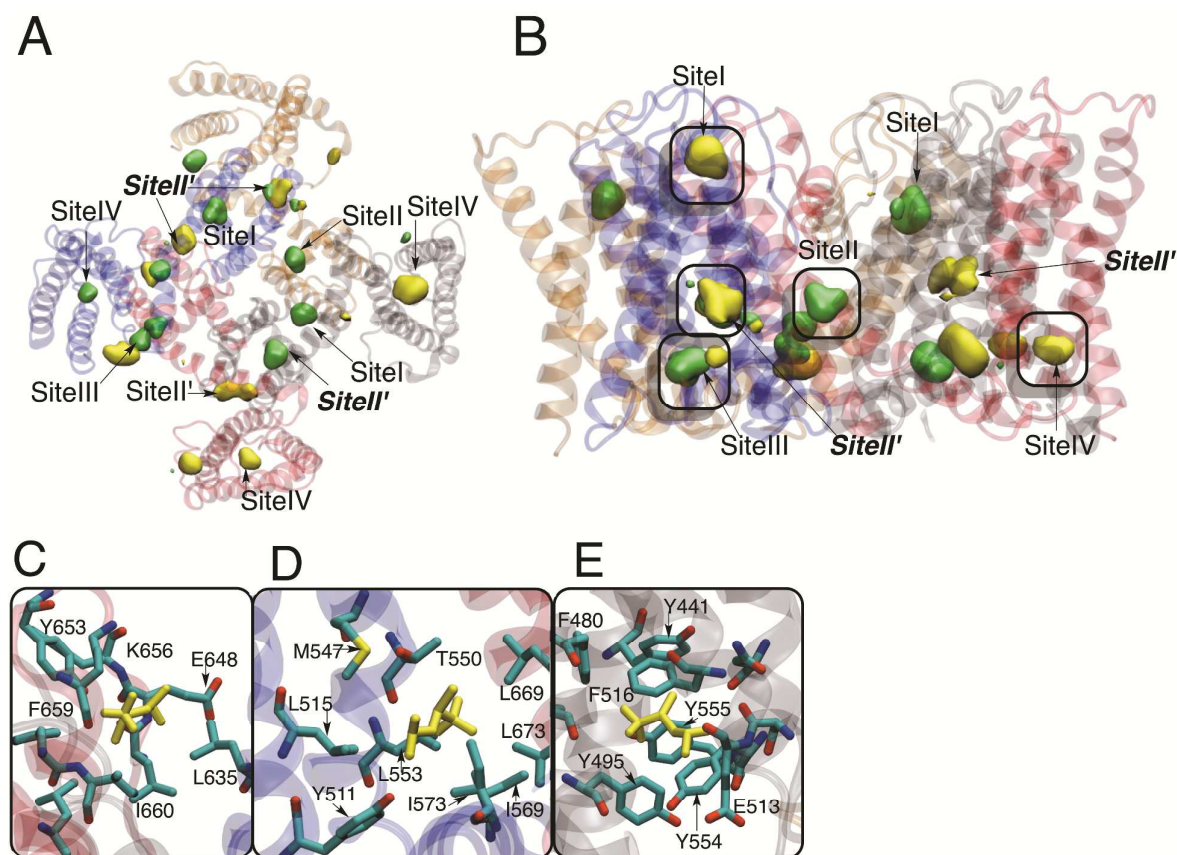


Figure 3. Population occupancy analysis for isoflurane (yellow) and chloroform (green) for the MD simulation using the full pore. (A) View of the system from the intracellular side in the XY plane, and (B) from a lateral perspective in the XZ plane of the membrane. Based on 500 ns of trajectory, an iso-0.2 contour criterion was employed for delimiting population, which shows iso-20% surfaces. (C, D, E) protein-anesthetic contacts within 4.5 Å distance cutoff, for key individual sites, in (C) for site I, (D) site II' and (E) site IV.

The iso-contour surfaces were used to generate a full probability distribution $Z(\xi)$ of anesthetics during the MD simulations, which, when Boltzmann-weighted, give the free-energy surface (FES) projected onto the xy ($\xi(x,y)$) and xz ($\xi(x,z)$) planes:

$$F(\xi) = -kT \log (Z(\xi)/Z_0) \quad \text{Equation 2}$$

This final distribution illustrated in Figure 4.A and 4.C for chloroform and isoflurane respectively is comparable to the iso-probability contour surfaces of chloroform (Figure 4.B) and isoflurane (Figure 4.D). Five binding sites are minima on the FES where the most long-lived binding sites (I, II' and IV) are separated by up to 3 kcal mol⁻¹ from the bulk. This result agrees with our estimate of binding free energy for chloroform and the free-energy gain for partition into the membrane. This projection also reveals the membrane-partitioned pathway to constitute a Minimum Free-Energy Path (MFEP) in agreement with the 4 kcal mol⁻¹ binding affinity of local anesthetic binding benzocaine in flooding simulations of Nav channels when compared to the bulk configuration.¹²

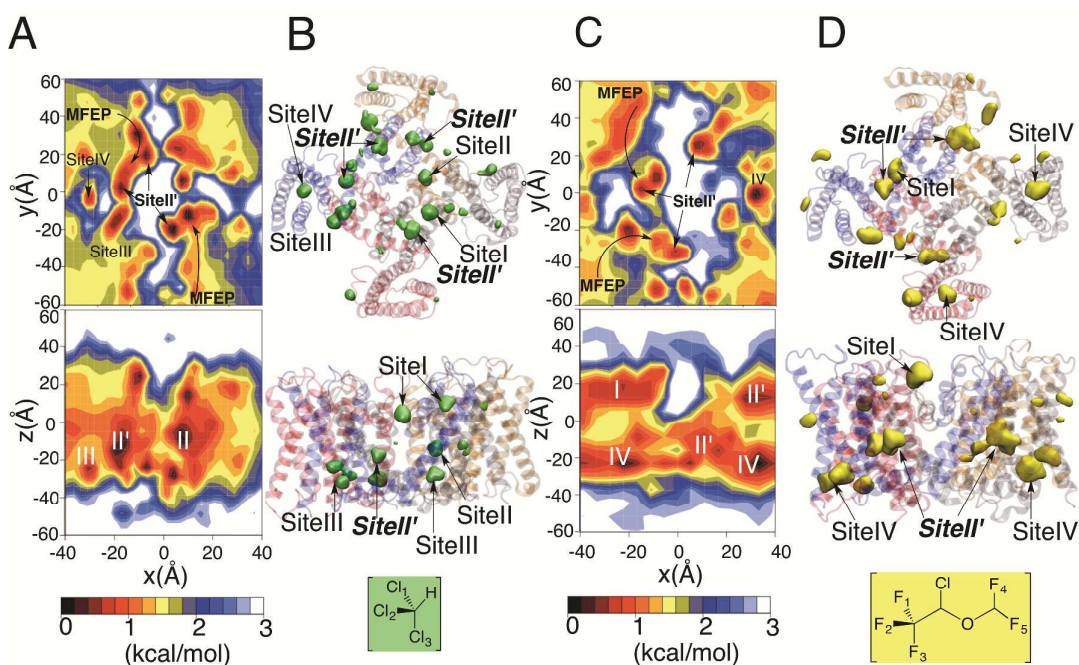


Figure 4. Classification of anesthetic binding sites according to their free-energy maps. XY and XZ surface projections of the free energy surfaces (FES) from the intracellular side for (A) chloroform and (C) isoflurane. The construction of the maps is detailed in the Methods section. Binding sites are labelled from I to IV. The minimum free energy pathway from the bulk solution to the binding site is indicated with black arrows. Based on two 500-ns of trajectory, an iso-0.2 contour criterion was employed for delimiting populations which shows iso-20% occupancy surfaces illustrated for (B) chloroform (green) and (D) isoflurane (yellow) using a top or side view of the channel with respect to the membrane normal.

The cost of moving isoflurane from the bulk solution to the polar head group interface of a POPC bilayer had previously been reported to be ≈ 4 kcal mol⁻¹ using umbrella sampling simulations.⁷⁰ Here, a 0.8- μ s well-tempered metadynamics simulation with funnel restraints was used to calculate the partition free energy from solution to the bilayer center. The restraint potential setup is given in Figure 5.A. The PMF for isoflurane and chloroform are reported in Figure 5.B, and the uncorrected ΔG of translocation obtained was -7.0 and -6.3 kcal mol⁻¹ for isoflurane and chloroform respectively. A geometric correction term (Equation 1) of magnitude -3.7 kcal mol⁻¹ was calculated which arises from the loss in conformational entropy in a bulk cylinder of radius $R_{\text{cyl}} = 1$ Å, and scaled by the standard concentration $C^0 = 1$ mol dm⁻³ = 1/1661 particles Å⁻³. The resulting corrected free-energies (ΔG°) were -3.3 and -2.6 kcal mol⁻¹ for isoflurane and chloroform respectively. Error bars were estimated by geometrically averaging the PMF at the plateau region in the last 100 ns of simulation. A standard deviation of 0.6 and 0.8 kcal mol⁻¹ for isoflurane and chloroform respectively is obtained. The final free energy of translocation, ΔG , between the bulk solution and the membrane center is -3.4 ± 0.6 and -2.5 ± 0.8 kcal mol⁻¹ for isoflurane and chloroform respectively.

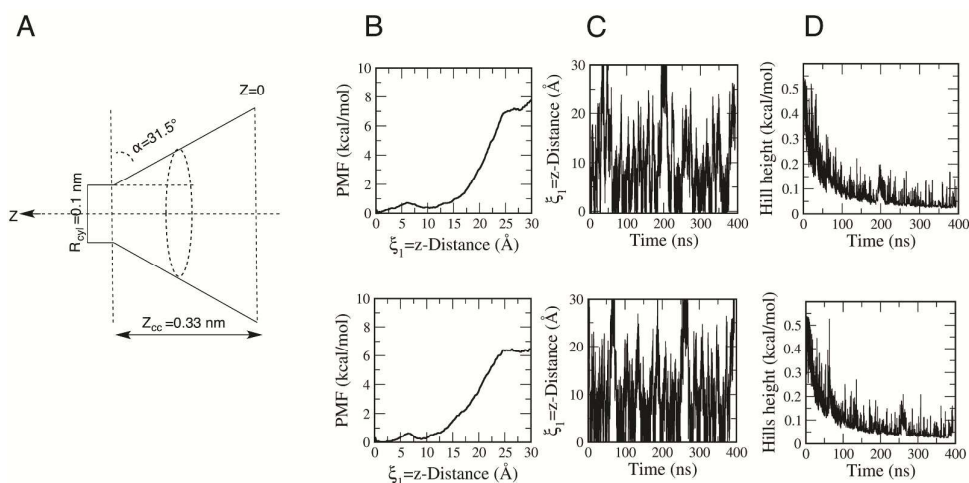


Figure 5. (A) Schematic funnel restraint setup. (B) Integrated 1D form of the potential of mean force (PMF) along ξ_1 (Z-axis position) from metadynamics simulations with a funnel restraint, (C) evolution of collective variables ξ_1 (Z-axis position) and (D) hills height. Upper panels correspond to isoflurane data and lower panels to chloroform data.

From the detailed residue analysis illustrated in Figure 3.C, the anesthetic binding site I is flanked by two TRPV1 monomers and located at the pore (P)-loop region. Isoflurane and chloroform share two hydrophobic contacts when bound in Site I of TRPV1, L635 and I660 (Supplementary Material Table S2). In addition, isoflurane also interacts with another hydrophobic residue (F659), a polar residue (Y653) and two charged residues (E648 and K656). In contrast, chloroform interacts with three hydrophobic residues (F638, L647 and L663) and only one charged (K639) residue (Supplementary Material Table S2). This difference can be ascribed to the ability of isoflurane to form hydrogen bonds between the halogen and the hydroxyl group of the side-chain of Y653, among others. The same site was experimentally identified and reported in TRPV1²³ and it was highlighted that residue E600 is essential for TRPV1 activation by protons and Y653 by heat and anesthetics. Isoflurane sampled extensively the same region reported experimentally, while chloroform diffused fast through the pore site, presumably aided by its smaller size.

Site II' is equivalent to the binding site described in the literature for TRPV1 activators,²⁹ notably capsaicin. The pocket has dimensions of ~ 20 Å (E570-L662; C α distance) by 12 Å (T550-I668; C α distance), sufficient to accommodate the capsaicin molecule. In our simulations, this site is sampled by isoflurane and chloroform that reach the site via the lipophilic entry mechanism and a minor direct transition from the pore loop site I. The membrane-partitioned anesthetic diffuses into the Vanilloid Binding Pocket (Site II'), which in this state of TRPV1 is open and solvent-exposed. In one case, entry into Site II' occurs through initial hydrophobic guidance across the protein-membrane interface with residues V318 and F522 of the S2-S3 transmembrane helices. In another case, diffusion into Site II' occurs via the opening at Site II mediated by L585 and I668, located at the interface between the membrane and the protein intrasubunit lumen. Both anesthetics make contacts with amino acid residues M547 and A665. In addition, isoflurane interacts with Y511, L515, T550, I569, L573 and L669

(Supplementary Material Figure S5.A) while chloroform interacts with a reduced number of amino acids: F591, I668 and L673 (Supplementary Material Figure S5.B). Alignment was made with the docked poses of capsaicin from previous work,³² depicted in Fig. S5. In addition to structural overlap with capsaicin poses, an overlap of volatile general anesthetic contacts was observed with reported capsaicin contacts in TRPV1³² for the down-conformation of capsaicin, Y511, L515, F543, M547, T550, A665, and L669, and for the up-conformation, Y511, L515, T550, F587, F591, I668, and L669. This site might constitute a potentiation or activation site of VGAs, akin to that of other TRPV1 agonists.

The side-pocket labelled site II located on the opposite side of the vanilloid binding pocket is flanked by the S4-S5 linker of one subunit, and the S6 P-loop of another subunit, similar to a propofol binding site reported for TRPA1.²⁰ Chloroform makes contacts with Y584, F580, M581, L664, T670, and I672 (Supplementary Material Table S2) while isoflurane sampled this site only transiently, moving into the adjoining site II' where it resided for up to 400 ns. Side-pocket site III was only sampled by chloroform and is a short-lived site, with contacts with Y565, R579, F580, V583, L674, M677 (Supplementary Material Table S2). This site is located in the space between S5 and S6 helices both from one subunit, and is a metastable site connecting Site II' of one subunit with Site II of another subunit (Figure 3). Finally, site IV is found in the space between helices S1, S2, S3 and S4, and is characterized by the presence of a majority of aromatic residues tyrosine and phenylalanine. Both isoflurane and chloroform make contacts with F488 and Y554. Isoflurane in addition interacts with Y441, Y495, F516, and N551, and a hydrogen bond can be formed between the isoflurane halogen atom and the hydroxyl hydrogen atom of Y495 and Y555, as well as hydrophobic interactions with the side chains of residues F488 and F516 (Supplementary Material Figure Table S2). Chloroform instead interacts with Y487, R491, F516, Y554 and Y555. The duration of these protein-ligand interactions is of hundreds of nanoseconds.

Complementing the MD simulations, a blind docking search on 500 representative snapshots of the TRPV1 transmembrane domain obtained from the holo-TRPV1 trajectory with capsaicin bound³² revealed that site II' in the vanilloid binding pocket was the most populated (26.7% for isoflurane, 58% for chloroform) while the pore loop site I was the second hit (9% for isoflurane, 13% for chloroform), as detailed in the Supplementary Material Table S3. This blind docking search was repeated on a TRPV1 trajectory without capsaicin bound revealing that site II' remained most populated for isoflurane (18%) while the pore loop site I became the most populated for chloroform (44.1%). These results suggest that anesthetics could indeed act as secondary TRPV1 agonists in the absence of capsaicin.

It is known that binding of capsaicin induces activation in TRPV1 by stabilizing the open conduction state.²⁹ The reported³² binding free-energy of capsaicin to TRPV1 is -10.6 ± 1.7 kcal mol⁻¹. It has been found that isoflurane does not bind in this site, and chloroform does not bind as strongly as capsaicin but it does so with a modest association found to be -2.0 ± 0.8 kcal mol⁻¹ (Supplementary Material

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3 Table S4), corresponding to a binding constant K_d value of 36 mM. This suggests that the mechanism of
4 action of anesthetics is different to the capsaicin mechanism of activation. It is known that volatile
5 general anesthetics either activate or sensitize the channel to activation.²⁴ Sensitization seems more
6 probable from the weak association of anesthetics in Site II'. One crucial difference from capsaicin is
7 the possibility of binding of more than one molecule of chloroform simultaneously in site II'. After one
8 binding event, during the simulation, it is observed that a second chloroform molecule enters the site.
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10 Therefore, the binding of multiple anesthetics at this site could compensate for their weak binding
11 affinity to enhance the sensitization effect on the channel. This is in agreement with recent work
12 suggesting that small agonists do bind in a concentration-dependent manner in order to enhance their
13 affinity as has been reported in the voltage-gated Kv1.2 ion channel.⁷¹ This can be partly explained due
14 to their size, smaller than capsaicin, and their promiscuous binding poses.
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19 **Discussion and Conclusions**

20 An understanding of the mechanisms by which general anesthetics modulate ion channels is essential to
21 clarify the underlying behavior of ion channels and their role in reversible immobilization and amnesia.
22 TRPV1 has distinct binding sites for its various agonists, and agonist binding is thus classed as
23 allosteric. While capsaicin binds to the intracellular vanilloid binding side-pocket, protons bind to the
24 extracellular outer-pore domain⁷²⁻⁷³ but both activate the channel.⁷⁴ Allosteric activation occurs by
25 preferentially stabilizing the open-activated conduction state over a closed-inactivated state. The exact
26 stoichiometry of TRPV1 agonist binding probed from patch clamp experiments revealed that while a
27 single capsaicin site is required to achieve a fully conducting TRPV1 pore, proton agonists require
28 binding to all four subunits to exert activating effects.²⁸ Currently, little is known about the sites at
29 which drugs exert their influence. In addition, there is an ulterior motive for studying the interactions of
30 volatile general anesthetics with TRPs which comes from the link between anesthesia and pain, and the
31 observation that general anesthetics, through direct actions at TRP channels, increase postsurgical pain
32 and inflammation.^{23, 75} In this study, we have focused on two general anesthetics chloroform and
33 isoflurane; it has been reported that chloroform binds in dorsal root ganglion neurons to induce TRPV1
34 activation while clinically relevant concentrations of isoflurane were shown to sensitize TRPV1 to
35 capsaicin and protons and reduce its threshold for heat activation.⁷⁵ Imaging studies also found that
36 chloroform and isoflurane act cooperatively with other TRPV1 agonists to induce a shift in the thermal
37 activation of TRPV1 toward lower temperatures in the presence of mM concentrations of them.⁷⁵ At
38 present, the general view is that chloroform and isoflurane could activate TRPV1 via similar
39 mechanisms²³ because they use overlapping sequence regions in the outer pore loop for activation,
40 namely (i) residues required for proton and anesthesia activation (E600), and (ii) residues required for
41 heat and anesthesia activation of TRPV1 (N628, N652, Y653).²³ The mechanism of TRPV1 activation
42 by anesthetics and the anesthetic interactions with TRPV1 remain unknown.
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3 Translocation of anesthetics through the membrane from the bulk solution has been characterized by
4 simulations with funnel-restrained metadynamics rendering a -3.4 ± 0.6 and -2.6 ± 0.8 kcal mol⁻¹ cost
5 for isoflurane and chloroform respectively. Once inside the membrane, anesthetics diffuse to the
6 protein. In this study, the anesthetics binding sites found in TRPV1 share common traits with four
7 binding sites reported for isoflurane in nAChR and GLIC channels: at the pore, inter-subunit, intra-
8 subunit, and annular sites,¹¹ as well as common traits with up to seven sites identified for the local
9 anesthetic benzocaine in a voltage-gated sodium channel (sites A to G).¹² The site is flanked by the S6
10 transmembrane helix and residue F203, and resembles the capsaicin site II' reported here, as well as
11 other six low affinities sites, including a pore site as well as an annular site in the outer transmembrane
12 domain region (similar to the annular site IV described in this work). Our computational work supports
13 a model where anesthetics occupy multiple sites with a total of five sites identified, in agreement with
14 experimental studies.^{23, 75} It has been proposed that chloroform and isoflurane could activate TRPV1 via
15 similar mechanisms²³ by using overlapping regions in the pore loop site I. This is in agreement with
16 reported studies showing that hydrophilic-polar interfaces bind volatile anesthetics.^{3-4, 22} Anesthetics
17 access these sites from the bulk solution through the cell membrane by diffusion. Anesthetic
18 partitioning converged after 0.2 μ s with 95% of isoflurane and 90% of chloroform present in the
19 membrane phase (Figure 2.B). The majority of bound anesthetic in TRPV1 entered through the
20 lipophilic phase (100% of bound isoflurane, 92.5% chloroform), while a minority of events were
21 observed for chloroform directly entering capsaicin site II' from the aqueous phase (Figure 2.A).
22 Finally, the way chloroform and isoflurane sample the inner-core of the protein is different. Chloroform
23 is smaller than isoflurane, and can access easily inner-protein regions that were banned for isoflurane
24 (sites II, and III) at least on the timescale of these simulations.

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36 Five binding sites for chloroform and three for isoflurane were characterized, the most long-lived of
37 which are separated by up to 3 kcal mol⁻¹ from the bulk (Figure 4.A and C). The simulations timescales
38 employed here were sufficient to observe single binding and unbinding events for isoflurane and
39 chloroform, but are not long enough to observe whether anesthetic binding is able to induce new
40 conformational states as opposed to only bind to the pre-existing states employed in our simulations.
41 These binding sites correspond to site II' for chloroform and sites IV and II' for isoflurane. Site I, in the
42 outer pore, shares protein-ligand contacts with the experimentally determined outer pore loop activation
43 site of TRPV1. The binding site of capsaicin in TRPV1 was also identified as an anesthetic site (Site
44 II'), although alchemical free-energy calculations suggest modest to no association when comparing the
45 free-energy of binding for anesthetics with the reported value for capsaicin which suggest an alternative
46 activation mechanism. The difference between site I and capsaicin site II' is the degree of
47 hydrophobicity of each binding site. Site II' is characterized by a hydrophobic, non-polar interface
48 (L515, T550, I569, L573, A665, and L669), while the pore site I is characterized by a hydrophilic polar
49 interface (E648, Y653, K656). The absence of significant structural rearrangements in the protein
50 following anesthetic binding is consistent with the weak interactions that are involved.

The data presented here only provides information of the location of binding sites with moderate affinity as demonstrated by persistent occupancy, and does not describe the effect of anesthetics on the channel global dynamics that ultimately produce the behavioral response so-called anesthesia. However, atomistic information about where these drugs bind provides a first step in the understanding of how they allosterically modulate the function of this family of ion channels, in particular sensitize the channel to activation by capsaicin or heat, which could help inform further experiments to help understand ligand binding and aid to develop and screen for new drugs.

Supporting Information. (1) Details of the MD simulation protocol; (2) RMSD profiles; (3) tables and distance profiles; (4) free-energy perturbation profiles and histograms. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Acknowledgements

The results of this research have been achieved using the DECI/PRACE resource Archer based in the UK at EPCC, Marconi based in CINECA in Italy, and SuperMUC based in Jülich in Germany with support from PRACE. C.J. thanks King's College London for a Graduate Teaching Assistant studentship.

Abbreviations

CNS, central nervous system; FEP, free energy perturbation; FES, free energy surface; GABA, gamma-Aminobutyric acid; GLIC bacterial (*Gloeobacter*) ligand-gated ion channel; MD, molecular dynamics; nAChR nicotinic acetylcholine receptors; Nav, voltage-gated sodium ion channel; NavAb, voltage-gated sodium ion channel from *Arcobacter butzleri*; TRP, transient receptor potential; TRPV1 transient receptor potential vanilloid member 1; VGAs, volatile general anesthetics.

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